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Spectral Studies on the Cooperative Binding Mechanism of Evans Blue to Poly(*N*-vinyl-2-pyrrolidone)

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The binding of Evans Blue (EB) to poly(*N*-vinyl-2-pyrrolidone) (PVP) was studied at two pH's 7.2 and 11.2 spectrophotometrically by absorbance change method. At pH 7.2, the absorption spectrum of EB changed unusually on adding PVP and this indicated the formation of two types of spectroscopically distinguishable PVP–EB complexes. At very low [PVP], a weak complex was formed which got converted into a strong complex at high [PVP]. Positive cooperativity was found to be operative in this conversion and in the strong complex at higher [PVP]. Scatchard, Hill, and Schwarz methods were employed to analyze this cooperativity and to determine the binding parameters. The analysis revealed: (i) the binding constants for the weak and strong complexes were of the order of 10^4 and 10^5 dm^3 mol^{-1} respectively; (ii) positive cooperativity at higher [PVP] was due to the interaction of neighboring binding sites on different polymer chains. At pH 11.2, only one type of complex was formed with higher affinity. But the binding behavior remained the same as at pH 7.2.

Evans Blue, a bisazo dye (C. I. No. 23860) is employed in blood volume determination. The basis behind this is its high binding affinity to plasma albumin. Resembling with serum albumin in many respects, for example, in binding behavior and in having amide linkage, the water-soluble synthetic polymer, poly(*N*-vinyl-2-pyrrolidone) (abbreviated as PVP) is physiologically and pharmaceutically an important macromolecule. In our earlier study,[1] the binding of the dye, Evans Blue (EB) to PVP was analyzed using a simple method, namely the Klotz's spectrophotometric method.[2] The significant finding of the study was that the dye binds mainly through electrostatic interaction. In the present work, employing absorbance change method[3] instead of the Klotz's method, we have attempted to investigate the cooperative binding mechanism, the binding forces and the nature of binding. Further, the unusual spectral change of EB with PVP, an interesting observation, has been found to be due to the formation of two types of spectroscopically distinguishable complexes. Thus all these features inspired us to make a thorough investigation of the system.

Experimental

Materials. PVP (average molecular weight=40000) and EB were purchased from Loba Chemie Indo Austranal Co., India. Urea, sodium chloride, and buffer reagents used were of analytical grade.

Method. The binding experiments were carried out at pH 7.2 in KH_2PO_4–Na_2HPO_4 buffer and at pH 11.2 in Na_2HPO_4–Na_3PO_4 buffer, both of 0.05 mol dm^{-3} concentration. All spectroscopic measurements were made at room temperature (ca. 25 °C) using Carl Zeiss Specord UV VIS spectrophotometer with matched 1 cm path length quartz cuvettes. The measuring pipettes and the cuvettes were prerinsed with the dye solution to reduce its concentration loss by adsorption onto the walls. Viscosities of PVP and PVP–EB solutions were measured at 30 °C with an Ubbelohde suspended level viscometer which has a flow time of 141 s for water.

For the binding studies, a dye concentration range of 7.5 to 25.0 μmol dm^{-3} was selected as admitted by the Beer-Lambert's law and PVP was employed in a wide concentration range from 1.11×10^{-7} mol dm^{-3} to 2.78×10^{-4} mol dm^{-3}, expressed in terms of polymer molecular weight. Urea and NaCl were used as additives in a range with maximum concentrations of 4.0 mol dm^{-3} and 1.6 mol dm^{-3} respectively.

Results and Discussion

Since the studies were conducted at two different pH's 7.2 and 11.2 and the binding behavior is almost the same at both the pH's, at first, the results obtained at pH 7.2 have been discussed in detail.

Quantification of Binding. The spectral shift of EB, at pH 7.2, from the λ_{max} of 609 nm to 639 nm on adding PVP forms the basis for the absorbance change method adopted in the present work. The absorbances of the dye were measured at 639 nm (at 609 nm when the pH is 11.2) (i) in the unbound or free condition (D_1), (ii) in the fully-bound condition (D_2) i.e., at the highest [PVP] and (iii) in the partially bound condition (D). Substituting all these values in Eq. 1, the bound dye concentration, C_B was calculated.

$$C_B = \frac{(D_1 - D)}{(D_1 - D_2)} \times C_T \qquad (1)$$

C_T is the total initial dye concentration, a known quantity. The conditions proposed for the application of the absorbance method are: (i) obeying of Beer-Lambert's law by both the free and the bound dye and (ii) the constancy of the molar extinction coefficient value of the complex with the variation of [dye]/[polymer] ratios. These have been found to be fulfilled in the present system and hence the dye-aggregation in the free as well as in the complexed states has been ruled out. Nonoccurrence of any blueshifts in the spectrum of EB with PVP also excludes, as pointed out by Takagishi et al.,[4] the association of dye molecules.

Scatchard method,[5] for which the equation is given

below, was followed to determine the binding parameters for the PVP-EB system.

$$\frac{r}{C_F} = nK - rK \qquad (2)$$

where r = the ratio, in moles, of bound dye to total polymer,
C_F = the unbound or free dye concentration,
n = the total number of binding sites per mole of polymer,
and K = the intrinsic binding constant, a measure of the strength of binding.

From the slope and intercept of the Scatchard plot, the binding parameters were calculated both in the absence and in the presence of the additives, 2.0 mol dm^{-3} urea and 0.4 mol dm^{-3} NaCl. Table 1 displays these values and Fig. 1 illustrates the representative Scatchard plots. Freedman and Johnson[6] have reported the K value of 4×10^5 dm^3 mol^{-1} for the human serum albumin-Evans Blue system at pH 7.3 in buffer-NaCl (0.12 mol dm^{-3}) mixture. This literature value is comparable with that of the PVP-EB system (K=1.03×10^5 dm^3 mol^{-1}) in 0.4 mol dm^{-3} NaCl, admitting the fact that we have used a higher [NaCl] which has certainly a binding-reducing effect.

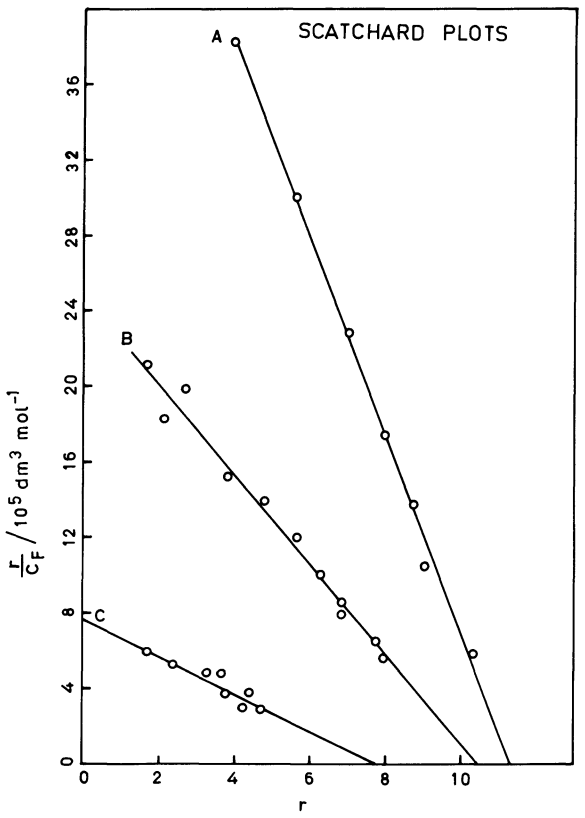


Fig. 1. Scatchard plots for the PVP-EB system at pH 7.2.
(A) PVP+2.0 mol dm^{-3} urea; (B) PVP only; (C) PVP+0.4 mol dm^{-3} NaCl, [PVP]=1.39 μmol dm^{-3} for B and C and 1.0 μmol dm^{-3} for A with varying [EB].

Spectroscopically Distinguishable Complexes. The spectrum of the dye, EB undergoes a peculiar shift (Fig. 2) when PVP is added from the lowest to the highest concentration of the range specified earlier. First, at very low [PVP], absorption at the λ_{max} of the free dye (609 nm or 16.42×10^3 cm^{-1}) decreases without any shift and it continues upto a [PVP] of 0.53 μmol dm^{-3}. In this condition, as evident from the isosbestic point at 635 nm, only two kinds of spectral species—the unbound dye and a weak PVP-EB complex—are existing in solution, maintaining equilibrium between themselves. When the added [PVP] is higher than 0.53 μmol dm^{-3}, two things happen: (i) spectral shifting to 639 nm (or 15.64×10^3 cm^{-1}) starts and (ii) the decreased absorption at 609 nm begins to increase with the disappearance of the isosbestic point. This drastic change indicates the

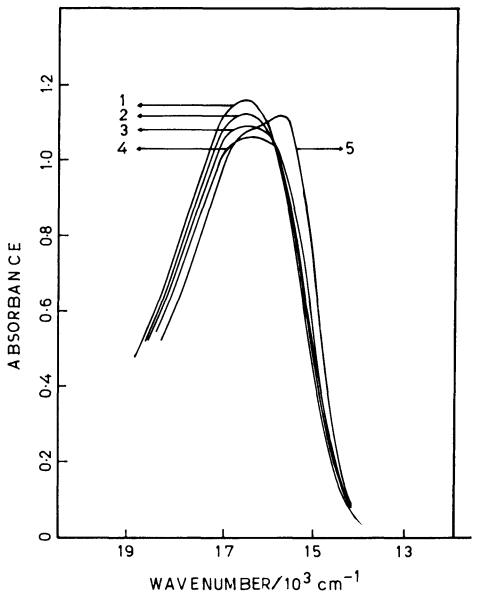


Fig. 2. Absorption spectra of EB and PVP-EB complex at pH 7.2. [EB]=2.0 μmol dm^{-3}.
1: EB only (λ_{max}=609 nm); (2—5): EB+PVP (λ_{max} for 5=639 nm) with [PVP] (in μmol dm^{-3})=0.2, 0.33, 0.53, and 2 respectively. Isosbestic point for the PVP-EB complex=635 nm.

Table 1. Binding Parameters[a] for the PVP-EB System at pH 7.2
[PVP]=1.39 μmol dm^{-3}; Temperature=25 °C

Parameter	PVP only	PVP[b]+2.0 mol dm^{-3} urea	PVP+0.4 mol dm^{-3} NaCl
n	10.36	11.24	7.57
$K/10^5$ dm^3 mol^{-1}	2.40	5.26	1.03

a) Calculated by the least-square method. b) [PVP]=1.0 μmol dm^{-3}.

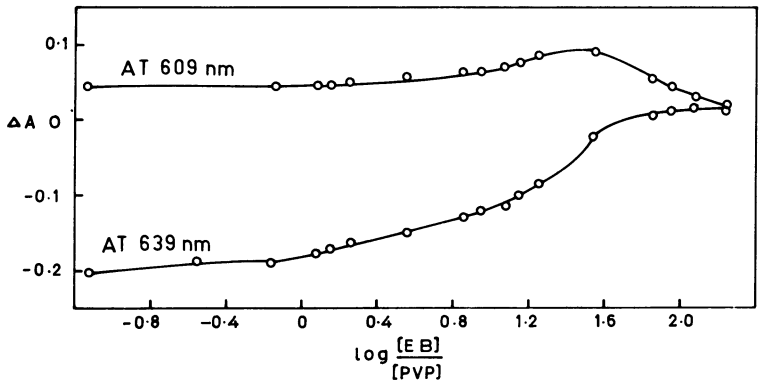


Fig. 3. Variation of absorbance difference ($\Delta A = D_1 - D$) with [EB]/[PVP] ratios at pH 7.2.
D_1=absorbance of the free dye; D=absorbance of PVP+EB mixture. [EB]=20.0 μmol dm^{-3}; [PVP]= 0.11 to 278.0 μmol dm^{-3}.

formation of the strong PVP-EB complex by the conversion of the weak one.

Figure 3 illustrates clearly the change in absorbance of the dye (20 μmol dm^{-3}) with [PVP] at the two wavelengths 609 nm and 639 nm. Upto [EB]/[PVP]$\geqslant$25, i.e, until log([EB]/[PVP]) reaches a value of 1.4, ΔA i.e., ($D_1 - D$) at 609 nm (ΔA_{609}) increases while ΔA_{639} first remains constant and then decreases. Since the changes of ΔA occur in two opposite directions upto the ratio of 25, it indicates the presence of two types of PVP-EB complexes in solution under equilibrium condition. Below the ratio of 25, change in ΔA_{609} as well as ΔA_{639} occurs in one direction only, i.e., a decrease and then constancy. This points out the existence of one complex i.e., the strong complex. Since all the experiments for determining the binding parameters in Table 1 were carried out at sufficiently high [PVP], the ratio [EB]/[PVP] is always less than 25 and hence the data correspond to the strong complex only. The designations 'strong' and 'weak' given to the complex, are purely relative and have been assigned only on the basis of the binding constant values.

Cooperativity and [PVP] Effect. Generally in ligand-polymer binding studies, cooperativity will be manifested at high [ligand]/[polymer] ratios only. Because, at this condition the number of available free sites will be much less than the free ligand concentration. Reeves et al.[7] have noticed cooperative binding at high dye concentrations only in 4-phenylazo-1-naphthol-2-sulfonate and the corresponding disulfonate-PVP systems. But on the contrary, in the present system cooperativity is explicit at the low [EB]/[PVP] ratio only i.e., a high [PVP] induces cooperativity. This is clearly seen from the convex Scatchard plots of Fig. 4 drawn for the set of a fixed [EB] with varying [PVP]. These plots obviously show that the operation of positive cooperativity is a function of [PVP]. This inference is again confirmed

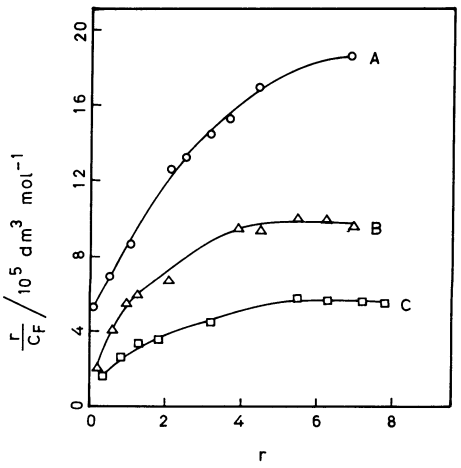


Fig. 4. Convex Scatchard plots for the PVP-EB system at pH 7.2. [PVP]=0.11 to 278.0 μmol dm^{-3}; [EB] (in μmol dm^{-3})=(A) 7.5; (B) 15.0 and (C) 25.0.

by the sigmoidal shape binding isotherm also i.e., r versus C_F plot (figure not shown). Figure 4 apparently shows that as the [EB]/[PVP] ratio is increased (e.g., at [EB]=25 μmol dm^{-3}), the extent of convexity is reduced. Therefore if we had used a still higher dye concentration, we would have obtained a concave Scatchard plot also indicative of negative cooperativity. But the experimental constraints did not allow us to do so. However in the reverse approach i.e. fixing the [PVP] constant and varying the dye concentration, an inverted 'U' shaped Scatchard plot (figure not shown) was obtained, suggesting the operation of both the positive and negative cooperativities.

The alternate inference from the afore-mentioned Scatchard plots is the ligand-mediated and ligand-facilitated association of polymer molecules. This possibility is ruled out on the grounds that the shapes and intersection of the theoretical Scatchard plots proposed by Cann[8] for the association of polymer

molecules do not coincide with that for the PVP-EB system. Moreover the values of the binding constants should be raised, if the polymer molecules were to associate through ligands. This was also not observed in the present system. Hence the above scatchard plots indicate only the operation of a 'mixed' cooperativity i.e., positive cooperativity at lower [EB]/[PVP] ratios and negative cooperativity at higher ratios. If the [PVP] is much less, the upward rising portion of the Scatchard curve disappears and only a linear down-ward plot (Fig. 1) is obtained, implying a simple model of binding.

Analysis of Cooperativity. Two methods—the well-known Hill method[9] and the Schwarz method[10]—have been employed for the analysis of cooperativity. Molyneux and Lentzos[11] have already applied these methods. For the use of both the methods an essentially accurate n value should have been known. The n value of 10.36 in Table 1 was verified by the difference spectrum method.[12] The difference spectrum of EB (20 μmol dm^{-3}) in varying [PVP] was recorded with the dye solution of same concentration as reference. The λ_{max} of the spectrum (661.4 nm) does not change upto a [PVP] of 2 μmol dm^{-3} and above this, it blueshifts to 651.5 nm. This observation demonstrates that one mole of PVP, on an average, can bind 10 moles of EB without any change in the nature of the complex. A higher [PVP] promotes a less polar environment around the already existing complex resulting in the blue-shift.

In Fig. 5 are shown the Hill plots constructed according to the Eq. 3.

$$\log\left(\frac{r}{n-r}\right) = \log K_a + n_H \log C_F \qquad (3)$$

Where K_a is the apparent binding constant and n_H the Hill coefficient. Figure 5 (Plot A) which exhibits clearly the operation of positive cooperativity in binding is the plot of the data obtained from a set of experiments with [EB]=7.5 μmol dm^{-3} and varying [PVP]. This plot is linear for 5 to 66% saturation of the binding. The curved portions at very low and very high degrees of saturation which reflect the single-site binding are extrapolated back to the abscissa to evaluate the first (K_1) and the last (K_n) ligand binding constants. These were used in calculating the

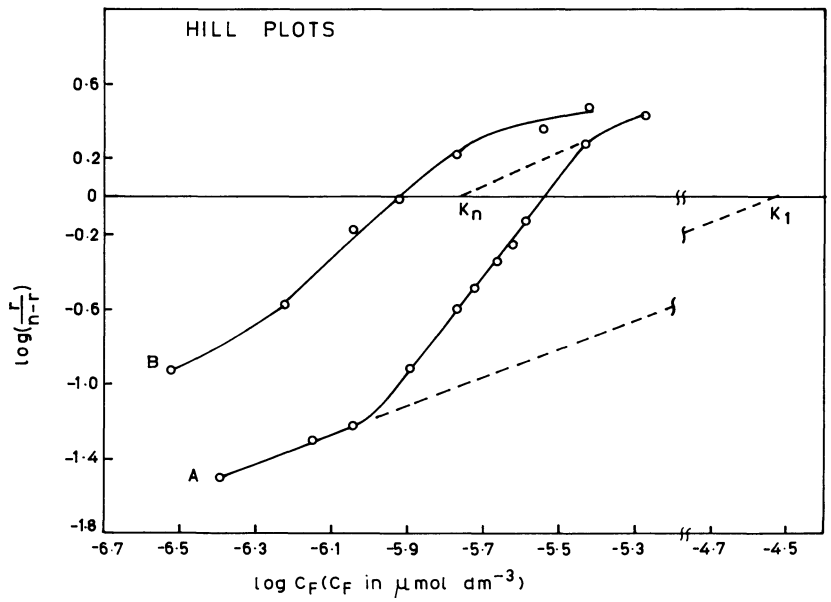


Fig. 5. Hill plots for the PVP-EB system.
(A): pH 7.2; [EB]=7.5 μmol dm^{-3}; [PVP]=0.28 to 22.2 μmol dm^{-3}. (B): pH 11.2; [EB]=12.5 μmol dm^{-3}; [PVP]=1.0 to 10.0 μmol dm^{-3}.

Table 2. Binding Parameters from the Hill Method at pH 7.2.
[PVP]=0.28 to 22.20 μmol dm^{-3}; Temperature=25 °C

[EB]	n_H	K_a	n_H[a]	K_1	n_H[b]	K_n	ΔG_{int}
μmol dm^{-3}		10^5 dm^3 mol^{-1}		10^5 dm^3 mol^{-1}		10^5 dm^3 mol^{-1}	kcal mol^{-1}
7.5	2.45	3.36	0.80	0.33	0.87	5.79	1.69
25.0[c]	2.32	1.08	—	—	—	—	—

a) and b) Values from the extrapolations at the very low and very high degrees of saturation respectively. c) Extrapolations not possible due to the experimental constraints.

interaction energy,[13] ΔG_{int} as given by Eq. 4.

$$\Delta G_{int} = RT \ln \frac{K_n}{K_1} \quad (4)$$

The values of the Hill parameters are presented in Table 2. When [EB] is increased from 7.5 μmol dm^{-3} to 25 μmol dm^{-3}, the Hill plot becomes linear for the entire data (3—76% saturation) and here K_a has a lower value. It is obvious from Table 2 that as [EB] is increased, the extent of positive cooperativity is diminished and thereby it supplements the Scatchard plots in Fig. 4. The values of the Hill coefficient imply that there is a constructive interaction between the two neighboring binding sites and that it is this interaction which promotes positive cooperativity. In the other experimental approach i.e., keeping the [PVP] constant and varying [EB], the Hill equation is not obeyed indicating the operation of a mixed cooperativity. PVP-EB system has an interaction energy of 1.69 kcal mol^{-1} (1 cal=4.184 J) or 163 cal/site which is less relative to the hemoglobin-O_2 system (3.0 kcal mol^{-1} or 750 cal/site).[13] This marked difference reflects the poor elevation of the binding strength in the PVP-EB system on each successive step of ligand binding.

Figure 6 shows the Schwarz isotherm at the [EB]=7.5 μmol dm^{-3}. The linearity of the plot suggests the obeying of the Schwarz equation given below:

$$\frac{(1-2\theta)\sqrt{C_F}}{\sqrt{\theta(1-\theta)}} = \frac{1}{\sqrt{K_0}} - \alpha C_F \sqrt{K_0} \quad (5)$$

Here θ = the fractional occupancy i.e., r/n,
K_0 = the intrinsic binding constant for a site when the sites on either side of it are unoccupied,
and α = the cooperativity parameter i.e., the increment in K_0 when one neighboring site is filled.

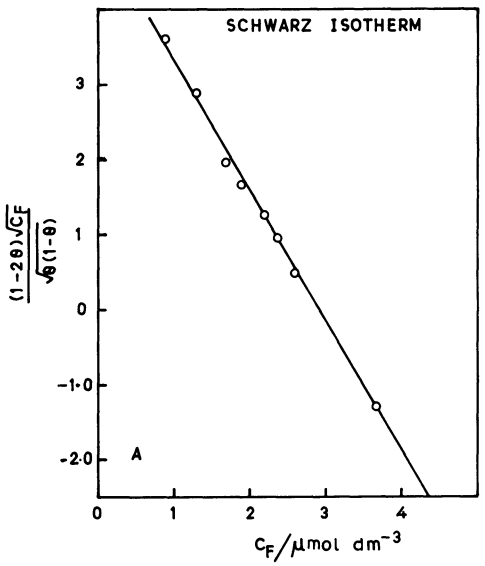


Fig. 6. Schwarz isotherm for the PVP-EB system at pH 7.2.
[EB]=7.5 μmol dm^{-3}; [PVP]=0.28 to 22.2 μmol dm^{-3}.

From the slope and intercept, α and K_0 were calculated and are given in Table 3 along with the Hill parameters. Table 3 shows that K_0 and K_1 are comparable as well as αK_0 and K_a, within the limits of experimental error. Thus the two methods, by virtue of their nature, provide the binding constant value (K_0 and K_1) of the PVP-EB weak complex also. Obeying of Schwarz model in the present system implies that at the lowest [PVP] (i.e. [EB]/[PVP]>25), when every polymer chain has only one EB molecule bound to the site, the complex exists as a weak one. But when further PVP is added, site-site interaction of two neighboring binding sites occurs leading to the conversion of the weak complex into the strong one.

Thus it is brought out from the above discussion that positive cooperativity is exhibited at two instances: (i) in the conversion of the weak to the strong complex and (ii) in the strong complex at higher [PVP]. But at the low [PVP] in the strong complex, no cooperativity is observed. Incorporating all these features, the following mechanism is proposed:

$$\underset{\text{(at the lowest concentration)}}{\text{PVP}} + \text{EB} \rightleftharpoons \text{Weak complex} \quad (6)$$

$$\text{Weak complex} + \text{PVP} \overset{\text{Cooperativity}}{\rightleftharpoons} \text{Strong complex} \quad (7)$$

$$\text{Strong complex} + \text{PVP} \overset{\text{Cooperativity}}{\rightleftharpoons} \text{Cooperative PVP-EB Strong complex} \quad (8)$$

Nature of the Complexes. The nature of the complexes formed depends on the structures of the polymer and the dye. PVP consists of nonpolar methylene and methine (CH) groups and the polar amide linkage. Due to the operation of keto-enol tautomerism, the heterocyclic N and the carbonyl O atoms acquire fractional positive and negative charges respectively to the extent of 0.37 units.[14] EB is a bisazo dye with two phenyl-substituted hydroxyl groups at

Table 3. Binding Parameters from the Schwarz and Hill Methods. pH=7.2; [EB]=7.5 μmol dm^{-3}; Varying [PVP]; Temperature=25 °C

Schwarz	Hill
$\frac{K_0}{\text{dm}^3\,\text{mol}^{-1}} = 3.89 \times 10^4$	$\frac{K_1}{\text{dm}^3\,\text{mol}^{-1}} = 3.33 \times 10^4$
$\alpha = 8.83$	
$\frac{\alpha K_0}{\text{dm}^3\,\text{mol}^{-1}} = 3.44 \times 10^5$	$\frac{K_a}{\text{dm}^3\,\text{mol}^{-1}} = 3.36 \times 10^5$

the ortho positions with respect to the azo groups. It also has hydrophilic ($-SO_3H$, $-NH_2$) and hydrophobic (biphenyl) groups in its structure (refer to Scheme 1). Two other structurally similar dyes, Trypan Blue (TB) and Benzopurpurin 4B (BP) have been chosen to determine the nature of the PVP–EB complexes. TB is a positional isomer of EB, the difference being in the position of the four sulfonato groups. In TB, two of the four sulfonato groups are very near to the azo groups i.e., at the ortho positions; the other two sulfonato groups are meta to the amino groups. This difference in position of the sulfonato groups between EB and TB reflects in the binding constant value also (as shown in the ensuing discussion). BP has the same basic skeleton as of EB and TB but without two hydroxyl and two sulfonato groups.

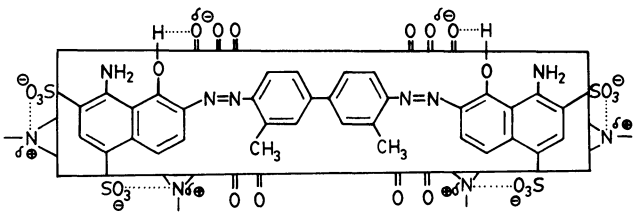
A comparison of the spectral behavior of these dyes at pH 7.2 shows that except BP, EB and TB exhibit, at first a decreased absorption at their respective λ_{max}'s with very low [PVP] and then a red shift at higher [PVP]. BP, on the other hand, shows only a redshift. At pH 11.2, the spectral behavior of EB (Fig. 9) gets changed from that at pH 7.2 and resembles that of BP. The reason for this is the ionization of the two phenolic hydroxyl groups at this alkaline pH. Correlation of these observations with the structures of the dyes leads to the interpretation that the initial decrease in absorption of EB and TB is mainly due to the -OH group of the dye and hence the formation of the weak complex. A similar decrease in absorption is also observed when NaCl is added to EB. From this, it is concluded that the formation of weak complex produces a polar environment around the dye, relative to that present with the dye alone. When urea is added to the weak complex the decreased absorption begins to increase at 0.5 mol dm^{-3} urea and at 4.0 mol dm^{-3}, the spectrum of the complex coincides with that of the free dye, suggesting the total collapse of the weak complex. Thus the urea effect, coupled with the above spectral studies indicates that the hydrogen bonding is the main force in the weak complex formation.

A brief description about the weak complex, emerging from the foregoing discussion is as follows: Since the [PVP] is very low, the polymer molecules would exist as single entities only. Hydrogen bonding interaction from the two -OH groups and the electrostatic interaction from the two sulfonato groups (far from the azo groups) favor its formation. Both the azo groups and the other two sulfonato groups (para to the amino groups) do not seem to involve in binding but get exposed to the solvent medium. Thereby they make the environment of the complex more polar.

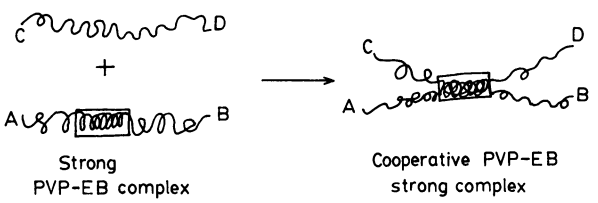
In the strong complex, the electrostatic interactions originating from the two remaining sulfonato groups, the dipolar and the hydrophobic interactions from the biphenyl part are the additional interacting forces. Now the azo group is present in a less polar environment as indicated by the redshift of the spectrum. Since the [PVP] is high, the PVP chains would be in somewhat coiled form and thereby make a less polar environment. All these features strengthen the PVP–EB attachment and hence the binding constant is enhanced by 7 times the value of the weak complex. Scheme 1 illustrates the model for the PVP–EB strong complex. In this model, the rectangle represents the PVP binding site with coiled chain.

The two sulfonato groups para to the amino groups are near to the azo groups, when compared to the other two sulfonato groups. The former sulfonato groups start interacting with PVP, only in the strong complex but not in the weak complex. This conclusion was made because of the following inference. The interaction from the anionic groups nearer to the azo group appears to contribute significantly to the binding affinity. The supporting evidences for this are: (i) $-O^-$ rather than -OH is the stronger interacting group, as revealed by the studies at pH 11.2; (ii) the PVP–TB strong complex has been observed to have a higher binding constant value than the PVP–EB strong complex. Since the azo group interaction is more pronounced in the strong complex only, the above logical conclusion was arrived at.

Further addition of PVP to the strong complex e.g., at a [PVP] of 10 or 20 μmol dm^{-3}, the polymer coils of the unoccupied sites interact hydrophobically, create a still less polar environment and thereby induces the site–site cooperative interaction. This is possible because at higher [PVP], intermolecular interactions such as polymer entanglement or interpenetration of polymer chains are unavoidable and therefore the site–site interaction is feasible. Creation of nonpolar environment and hence the positive cooperativity is proved by (i) change in λ_{max} of the difference spectrum, (ii) effect of urea on binding at higher [PVP] (discussed



Scheme 1. PVP–EB strong complex.



Scheme 2. Cooperative interaction in the strong PVP–EB complex.

Table 4. Viscosity of PVP and PVP-EB Complex at pH 7.2. 0.05 mol dm^{-3} Buffer; Temperature=30 °C [EB]=25.0 μmol dm^{-3}

Sample	Intrinsic viscosity
	ml g^{-1}
PVP	20.62
PVP+EB	21.56

later) and (iii) cooperativity as a function of [PVP] previously illustrated by Scatchard and Hill plots. Scheme 2 pictorially represents the cooperative interaction. In this model AB and CD are two different polymer chains and the rectangle denotes the bound dye molecule.

The question of positive cooperativity due to the interaction between adjacent sites on the same polymer chain rather than on different polymer chains is ruled out because, if it were the case, we should have observed cooperativity at the very low [PVP] also. But it is not the case. Further cooperativity of sites on the same polymer chain would facilitate the dye binding on a single macromolecule resulting in the overall conformational change. This was also not encountered as indicated by the viscosity data in Table 4. Thus from the foregoing arguments, it is clear that the observed positive cooperativity in the strong complex is exclusively due to the interaction between the sites on different polymer chains. The viscosity data also reveal the absence of coulombic repulsion between the bound dye molecules and hence the chain elongation.

Influence of Additives on Binding. In our previous article[1] we have reported that both urea and NaCl reduce the binding constant values at higher [PVP]. But at lower [PVP], urea enhances the binding constant while NaCl decreases it. In other words, urea plays a dual role while NaCl has only one effect i.e., the binding reducing effect. The trend at lower [PVP] is observed in the present work also (Table 1). The explanation for this is based on the effect of these additives on the formation of weak and strong complexes. Decrease in absorbance at 609 nm (D^{609}) and the increase in absorbance at 639 nm (D^{639}) of the PVP-EB mixture, relative to the absorbance of the free dye (D_1) have been considered as to signify the formation of the weak and strong complexes respectively. Therefore if the addition of urea or NaCl decreases the value of D^{609}, it is considered as to favor the formation of the weak complex. If D^{609} is increased, it indicates the inhibition of the weak complex formation. Oppositely, the increase in D^{639} represents the facile formation of the strong complex and the decrease in D^{639}, the inhibition.

Figure 7 exhibits clearly the urea effects (4.0 mol dm^{-3}) in terms of the plot ΔA or $(D-D_1)$ versus [EB] at both the wavelengths. At the [PVP] of 0.4 μmol dm^{-3},

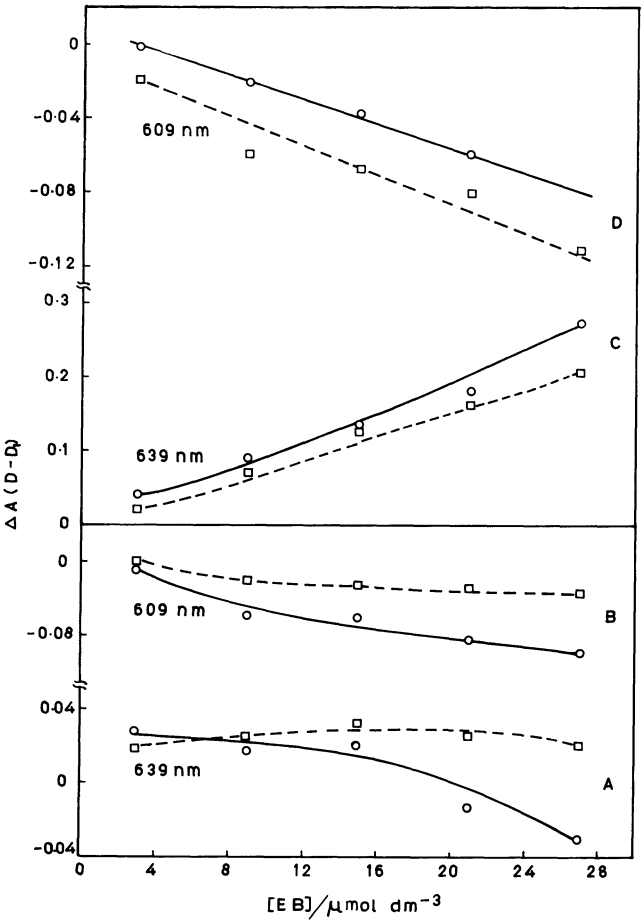


Fig. 7. Difference in absorbance, ΔA and urea effects at pH 7.2, [urea]=4.0 mol dm^{-3}.
For A and B [PVP]=0.4 μmol dm^{-3} and for C and D [PVP]=50.0 μmol dm^{-3}. Solid line: PVP+EB; dotted line: PVP+EB+urea. D=absorbance of the complex; D_1=absorbance of the free dye; $\Delta A = D-D_1$.

addition of urea to PVP-EB mixture increases the absorbance at both the wavelengths. This means that the strong complex formation is favored at the decay of the weak complex. But at higher [PVP] (50 μmol dm^{-3}), urea produces the opposite effect i.e., absorbances are decreased at both the wavelengths. This decrease in absorbance at 609 nm of the PVP+EB mixture by urea (the set of two lines D in Fig. 7) should not be taken as an indication of the formation of weak complex. Because, at this higher [PVP], as already stated, only one type of complex i.e., the strong complex alone can exist. Therefore, this decreased absorbance at 609 nm, as already discussed in the nature of the PVP-EB weak complex, could be considered only as reflecting a more polar environment around the PVP-EB strong complex. Thus at higher [PVP], the strong complex formation is hindered by urea, as a result of the change in polarity of the binding environment.

The underlying principle for both the urea effects is

the water-structure breaking action. It has been already established that for the effective interaction of different polar groups in aqueous solution in close proximities, the water structure around these groups is an inhibiting factor. As already mentioned, in the conversion of weak to strong complex, additional electrostatic and dipolar interactions from the sulfonato, azo, and biphenyl groups begin to operate. Therefore the breaking of water structure around the hydrophilic groups by urea in the weak complex favors an effective interaction between PVP and EB and facilitates the conversion of weak to strong complex. Conversely at higher [PVP], the nonpolar interactions operating between different polymer chains in the cooperative PVP–EB strong complex is disfavored to some extent by urea through the destruction of iceberg structure of water. This type of inhibition of hydrophobic interactions by urea has already been noticed in many studies.[15] This effect leads to the exposure of the PVP–EB strong complex to a relatively less nonpolar environment and hence reduces the binding affinity. Now the urea effect is summarized as follows:

$$\text{At lower [PVP] : Weak complex} \xrightarrow{\text{Urea}} \text{Strong complex} \quad (9)$$

$$\text{At higher [PVP] : Strong complex (in nonpolar environment)} \xrightarrow{\text{Urea}} \text{Strong complex (in less nonpolar environment)} \quad (10)$$

Thus these two types of effects are responsible for the dual role played by urea.

On the contrary to urea, NaCl decreases the absorbances of the complex at both the wavelengths at all PVP concentrations. This means that the strong complex formation is hindered by NaCl, irrespective of the [PVP] employed. Since the present work is mainly concerned with the strong complex, the observed effect of NaCl is only a reduced binding at all PVP concentrations. This effect may be caused in two ways: (i) the water-structure breaking action and (ii) the interaction of Na^+ ions with the hydrophilic groups e.g. the sulfonato and the azo groups. The latter way appears to be the predominant one because, the strong complex formation to which the contribution from the electrostatic interaction is very much, is hindered.

Binding Forces. The forces involved in binding need not be discussed in detail because the operation of electrostatic forces, hydrogen bonding and hydrophobic interaction in the system has already been mentioned. Besides these, the dipolar forces may also operate between the easily polarizable aromatic

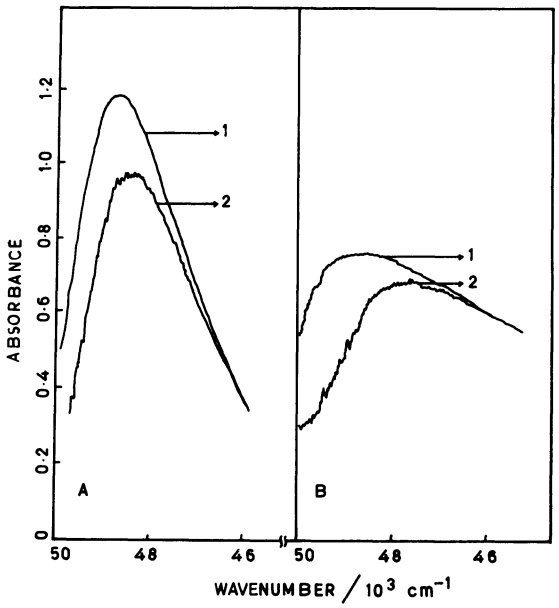


Fig. 8. UV spectra of PVP and EB at pH 7.2. [PVP]=1.0 μmol dm^{-3} and [EB]=20.0 μmol dm^{-3}. A: (1) Spectrum of PVP alone (λ_{max}=205.2 nm); (2) Spectrum of PVP in the presence of EB (λ_{max}=206.4 nm). B: (1) Spectrum of EB alone (λ_{max}=205.3 nm); (2) Spectrum of EB in the presence of PVP (λ_{max}=210 nm).

moieties and the >C=O group in PVP. Figures 8A and 8B illustrate respectively the UV spectra of PVP and EB in the presence of the other component. The $n\rightarrow\pi^*$ transition of both the components get redshifted with decreased intensity. This clearly demonstrates the creation of a less polar environment and in turn the mutual delocalization of electrons and the ground state stabilization. All these aspects facilitate the polar interactions.

Studies at pH 11.2. At this alkaline pH, the two phenolic hydroxyl groups are ionized ($pK_a \simeq 10$) and the dye acquires 6 negative charges. Unlike at pH 7.2, only one type of complex is formed at pH 11.2. This is shown in Fig. 9 with the spectral change in only one direction. The free dye has λ_{max} at 570 nm and the bound dye at 609 nm. Because of this spectral shift, a very attractive instantaneous color change can be seen, when one drop of PVP solution is added to the dye solution. The isosbestic point at 573.4 nm is an additional proof for the existence of only one type of complex. Since the λ_{max} of the bound dye coincides with that of the free dye at pH 7.2 (609 nm), it is concluded that the EB molecule in the PVP–EB complex, at the alkaline pH, should be present with the two phenoxide anions involved in ion-pair formation with the fractionally positively charged N atom of the pyrrolidone ring. This ion-pair formation is very much facilitated because of the nonpolar environment provided by the PVP molecular chains. Such ion-pair formation as this, has already been

Table 5. Binding Parameters for the PVP-EB System at pH 11.2. Temperature=25 °C

Scatchard method[a]	Hill method[b]	Schwarz method[b]
K=8.14×10^5	K_a=8.17×10^5	K_0=2.78×10^5
n=11.49	n_H=1.75	α=2.91
		αK_0=8.11×10^5

All the binding constants are in dm^3 mol^{-1} unit. a) [PVP]=1.0 μmol mol^{-3} with varying [EB]. b) [EB]=12.5 μmol dm^{-3} with varying [PVP].

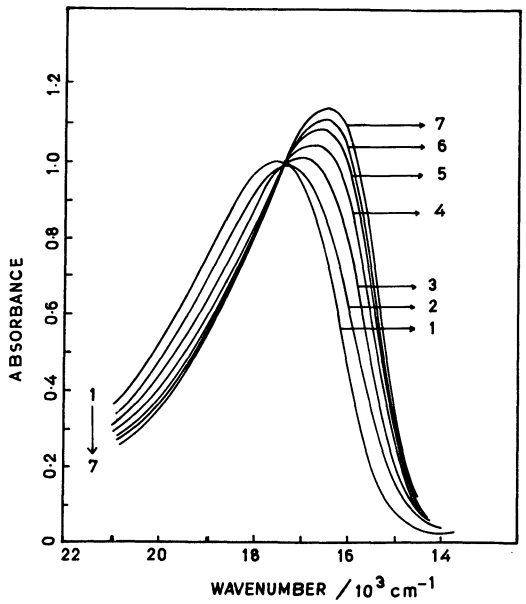


Fig. 9. Spectra of EB and PVP-EB complex at pH 11.2.
[EB]=27.5 μmol dm^{-3}. (1) EB only (λ_{max}=570 nm); (2—7): EB+PVP (λ_{max} for 7=609 nm) with [PVP] (in μmol dm^{-3})=0.5, 1, 2, 5, 10 and 25 respectively. Isosbestic point for the PVP-EB complex=573.4 nm.

reported in the bovine serum albumin-Methyl Orange system.[16]

Eventhough only one type of complex is formed, the entire binding behavior is the same as in pH 7.2. The binding parameters determined from various methods are given in Table 5. Extrapolations in the Hill plot (plot B; Fig. 5) to evaluate K_1 and K_n were not possible under the experimental conditions at pH 11.2. The dye has higher affinity at this pH and this is due to the involvement of two more electrostatic interacting sites ($-O^-$ ions) in binding. The cooperativity parameter α is one-third of the value at pH 7.2 and this may be due to the absence of weak complex in initial stage. Incorporating all these, the following mechanism is put forward.

$$\text{PVP} + \text{EB} \rightleftharpoons \text{Complex} \quad (11)$$

$$\text{Complex} + \text{PVP} \xrightleftharpoons{\text{Cooperativity}} \text{Cooperative PVP-EB complex} \quad (12)$$

Comparison of the binding studies at the two pH's points out that the two phenolic hydroxyl groups play the key role in establishing the binding behavior in the PVP-EB system. From the whole study the conclusion arrived at is that although a synthetic polymer, PVP can be placed in equal rank with the protein molecules as the former is also able to exhibit a cooperative mechanism like the biomacromolecules in the binding of ligands.

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